Stimulation of Humoral and Cellular Antibody Formation in Mice by Poly I_r:C_r[1] (34469)

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The synthetic double-stranded RNA, polyriboinosinic–polyribocytidylic acid (poly I_r:C_r), has been show to be a potent inducer of interferon (1). Animals treated with poly I_r:C_r have been shown to exhibit high levels of circulating interferon and thus were shown to be protected against the lethal effects of several non-oncogenic and oncogenic viruses (1, 2). Poly I_r:C_r has also been reported to induce antitumor activity in mice against several viral and nonviral induced transplantable tumors (3, 4). The mechanism by which poly I_r:C_r induces antitumor activity in mice is not known, but is not thought to be attributable solely, if at all, to interferon (3).

Braun and Nakano reported that complexes of polyadenylic–polyuridylic acid (poly A:U), enhanced the early rate of increase in number of antibody-forming spleen cells in mice immunized with sheep erythrocytes (5). This observation clearly establishes the adjuvant activity of poly A:U in mice. This report concerns the ability of poly I_r:C_r to induce a state of immunological hyperresponsiveness in mice.

Materials and Methods. Mice. Adult C_{57}Bl/6 male and female mice, 6–10 weeks old, between 20–25 g, were obtained from the animal production section of the National Institutes of Health. Although this is an isogenic strain, the female of this strain rejects an isograft from male donors. This rejection is thought to be due to a histocompatible antigen (H-Y transplantation antigen) present in male skin which is determined by a locus on the y chromosome (6). Mice of the same age were employed for antibody production and isograft studies.

Grafting technique. The grafting technique employed in this study is essentially the same as that employed by Howard *et al.* (7). Briefly, tail skin grafts from C_{57}Bl/6 male mice were placed on the thoracic wall of female C_{57}Bl/6 mice and covered with Vaseline-impregnated gauze and a plastic cast. Casts were removed 7 days after grafting and the grafts were inspected daily for signs of rejection. Grafts were considered to be rejected at the first signs of hemorrhage or induration.

Poly I_r:C_r. Polyriboinosinic-polyribocytidylic acid was purchased from Grand Island Biological Company (Gibco), Grand Island, New York. A stock concentration of poly I_r:C_r containing 10 mg/ml was stored at 4° and diluted in phosphate buffered saline (PBS), pH 6.0, to obtain the desired concentration of poly I_r:C_r to be used in the experiments.

Antigen. Sheep red blood cells (S-RBC) obtained commercially from Microbiological Associates, Inc., Bethesda, Md., were washed three times in 0.85% NaCl, resuspended in saline, and standardized photometrically to a concentration of 5×10^8 cells/ml. Mice were immunized by intraperitoneal inoculation of 10^8 sheep cells in a 0.2-ml volume.

Hemolytic antibody titration. Mice were bled at various intervals following immunization with S-RBC. Individual serum samples were obtained from 5 to 8 mice/time interval, heat inactivated at 56° for 30 min and stored at —20° until titrated. The titer of hemolytic antibody in each serum sample was obtained by modification of the method described by Taliaferro and Taliaferro (8). Briefly, serial twofold dilutions of serum, 1:25 through

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1:1600, in 0.5-ml volumes were made in Veronal buffered saline (pH 7.3–7.4). An equal volume of 2% S-RBC was added to antiserum dilutions and allowed to stand at room temperature for 15 min for sensitization. Six-tenths ml of sensitized S-RBC was added to tubes containing $5C'_{50}H$ units of guinea pig complement in 0.3-ml volume and 0.6 ml of Veronal buffered saline (VBS). Tubes were shaken and incubated at 37° for 30 min. Five-tenths ml of VBS was added to each tube and tubes were then centrifuged at 1000*g* for 10 min. Photometric reading of each tube at a wavelength of 500 mμ were taken on a Coleman Jr. photometer and compared to the photometric reading of a 100% hemolysis blank to determine the percentage hemolysis. Percentage hemolysis vs. dilution of serum were plotted on probit graph paper to determine the titer of hemolytic antibody. The hemolytic antibody titer is expressed at the number of 50% hemolytic units (H_{50})/ml of serum. The peak titer of hemolytic antibody consistently occurred 7 days after immunization with S-RBC.

Statistical methods. The *p* values were obtained by comparing each treatment group with control group by the *t* test (9) and Wilcoxon-Mann-Whitney test (10). The confidence intervals for the median were obtained by a method described by Dixon and Massey (11).

Results. Sheep red blood cell hemolytic antibody formation in mice treated with poly I_r:C_r. Mice were inoculated intraperitoneally with a dose of 200 μg of poly I_r:C_r at different time intervals before and after S-RBC immunization. Data in Fig. 1 show that 7 days after immunization all the regimens of poly I_r:C_r treatments employed resulted in the formation of increased titers of S-RBC hemolytic antibodies over S-RBC controls. The most significant enhancement of hemolytic antibody titer (3.1-fold) over controls occurred in mice simultaneously inoculated with poly I_r:C_r and S-RBC. The increased titers which occurred at 7 days were not reflected in any marked earlier appearance of higher titer of antibodies, nor were the titers maintained significantly higher at 15 days.

TABLE I. The Effect of Treatment of Mice with Various Doses of Poly I_r:C_r on Hemolytic Antibody Formation.

Treatment			
Dose of poly I_r:C_r (μg/mouse)[a]	Antigen[b]	Hemolytic antibody[c] titer (H_{50}/ml)	p[d]
1	S-RBC	386	>0.10
10	S-RBC	592	<0.01
50	S-RBC	507	<0.05
100	S-RBC	787	<0.001
200	S-RBC	728	<0.001
—	S-RBC	292	—

[a] Poly I_r:C_r was inoculated intraperitoneally in 0.2-ml volume at time of S-RBC immunization (D_0).

[b] Mice were inoculated intraperitoneally with 10^8 S-RBC on D_0 and bled 7 days after immunization.

[c] Hemolytic antibody titer represents mean of hemolytic antibody titer of 5 mice. Standard error of the mean is 73.3 units, with 24 *df*.

[d] The *p* values were obtained by a *t* test.

Effect of treatment of mice with different doses of poly I_r:C_r on hemolytic antibody formation to S-RBC. Since simultaneous injection of poly I_r:C_r resulted in the formation of the highest titer of hemolytic antibody, this treatment schedule was employed to ascertain the lowest concentration of poly I_r:C_r which would enhance hemolytic antibody formation. Results in Table I show that treatment of mice with 100 μg of poly I_r:C_r resulted in a maximum increase in titer of hemolytic antibodies over S-RBC controls (approximately 3-fold). Treatment of mice with 200 μg of poly I_r:C_r did not result in additional enhancement of hemolytic antibody titer beyond that induced in mice treated with 100 μg of poly I_r:C_r. The results obtained in mice injected with 10 or 50 μg of poly I_r:C_r indicated that enhancement of hemolytic antibody formation occurred, which would represent a response to dose of poly I_r:C_r. The increase in hemolytic antibody attained in mice injected with 1 μg of poly I_r:C_r was not considered significantly greater ($p > 0.10$) than that observed in control mice. The results of this study show that the minimum dose of poly I_r:C_r which will enhance hemolytic antibody titers signifi-

cantly higher than control values is 10 μg.

In subsequent studies single-stranded RNA, poly I_r, or poly C were tested for possible enhancing effects. Injection of 200 μg of either polynucleotide alone did not enhance hemolytic antibody formation in mice immunized with S-RBC. The findings suggest that only the complexed polynucleotides exhibit adjuvant activity in mice.

The effects of poly I_r:C_r on isograft survival. Female C_{57}Bl/6 mice treated at different time intervals with 200 μg of poly I_r:C_r received grafts of tail skin from C_{57}B1/6 male mice. Results in Table II show that the median survival time (MST) of control grafts was 23 days. Earlier rejection of skin grafts occurred in mice which received treatment with poly I_r:C_r. Mice which were grafted and received one treatment with poly I_r:C_r (200 μg) on the same day rejected their grafts 13 days prior to controls. A similar response was attained in mice which received poly I_r:C_r 10 days prior to and again on the day of grafting, and in mice which received 5 treatments (D_{-3}, D_{-1}, D_0 D_{+2}, D_{+7}). The earlier time of skin graft rejection in these treated groups was highly significant ($p < 0.005$). The group of mice which were injected with poly I_r:C_r 10 days prior to receiving skin grafts rejected their grafts at a MST

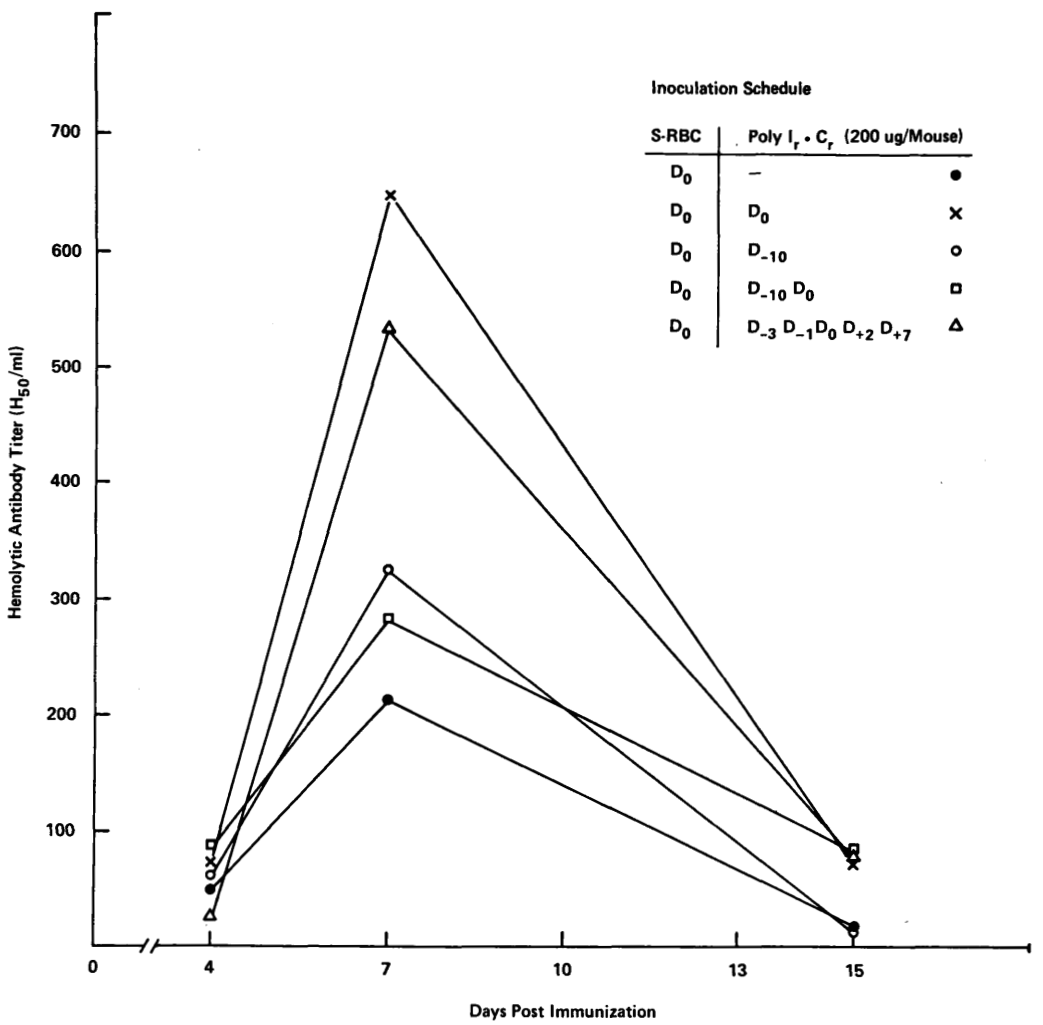

FIG. 1. Hemolytic antibody formation in poly I_r•C_r-treated mice immunized with sheep erythrocytes. D_0 = day of sheep erythrocyte inoculation.

TABLE II. The Effect of Poly I_r:C_r Treatment of Female C_{57}Bl/6 Mice on the Survival Time of Isografts from Male C_{57}Bl/6 Donor.

Poly I_r:C_r[a] treatment schedule	No. of mice with graft	MST[b] of graft (days)	95% confidence interval for MST of graft	p[c]
D_{-10}	14	24	$20 \leq MST \leq 26$	>0.10
D_0	9	10	$10 \leq MST \leq 17$	<0.005
D_{-10}, D_0	7	10	$8 \leq MST \leq 21$	<0.005
D_{-3}, D_{-1}, D_0, D_{+2}, D_{+1}	7	10	$8 \leq MST \leq 16$	<0.005
None	10	23	$18 \leq MST \leq 34$	—

[a] Mice inoculated intraperitoneally with 200 μg of poly I_r:C_r on each day of treatment. D_0 = day of grafting, while D_{-10} = 10 days before grafting.

[b] MST = median survival time.

[c] The Wilcoxon-Mann-Whitney test was employed to obtain p values.

similar to nontreated controls. The excellent effects achieved with the treatment of poly I_r:C_r, at the time of grafting, indicates that one treatment was sufficient to enhance graft rejection.

Discussion. The results reported herein demonstrate the adjuvant activities of poly I_r:C_r in mice. These findings confirm the observation of Braun and Nakano that double stranded polynucleotides stimulate antibody formation in mice to sheep erythrocytes (5). In addition, our results show that poly I_r:C_r treatment significantly reduced the survival time of isografts in mice. Thus, the enhanced immunological responsiveness induced by poly I_r:C_r to two antigens, S-RBC and H-Y transplantation antigen (isografts), show that this polynucleotide copolymer is capable of stimulating both humoral and cellular antibody formation.

The responses achieved, when the time of injection and the dose of poly I_r:C_r was varied, were of interest. Simultaneous administration of poly I_r:C_r and antigen resulted in maximum increase in antibody formation. A single dose was sufficient for this stimulation. A concentration of poly I_r:C_r as low as 10 μg was sufficient to significantly enhance antibody formation to S-RBC. Of particular interest were the results achieved with skin isografts. The very early rejection of the grafts (10 days) indicate that a very strong cellular immunity was established in grafted mice treated with one dose of poly I_r:C_r.

The mechanism(s) by which poly I_r:C_r protects mice against viral and nonviral induced transplantable tumors is, as yet, not clearly defined. The ability of poly I_r:C_r to stimulate antibody formation must also be considered as an important factor in the mechanism(s) by which poly I_r:C_r exerts its antitumor effects.

Summary. Polyriboinosinic–polyribocytidilic acid stimulated formation of hemolytic antibodies in mice immunized with sheep erythrocytes. Similarly, poly I_r:C_r treatment of mice resulted in a marked reduction in survival of isografts. Temporarily, administration of poly I_r:C_r at the time of exposure of mice to antigen resulted in optimal enhancement of humoral and cellular antibody formation. Optimal enhancement of both humoral and cellular antibody formation was induced by 100–200 μg of poly I_r:C_r, while significant stimulation of hemolytic antibody formation was induced by a lower dose of 10 μg of poly I_r:C_r.

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1. Field, A. K., Tytell, A. A., Sampson, G. P., and Hilleman, M. R., Proc. Natl. Acad. Sci. U. S. **58**, 1004 (1967).

2. Sarma, P. S., Shin, G., Neubauer, R. H., Baron, S., and Huebner, R. J., Proc. Natl. Acad. Sci. U. S. **62**, 1046 (1969).

3. Levy, H. B., Law, L. W., and Rabson, A. S., Proc. Natl. Acad. Sci. U. S. **62**, 357 (1969).

4. Zelenick, L. D. and Bhuyan, B. K., Proc. Soc. Exptl. Biol. Med. **130**, 126 (1969).

5. Braun, W. and Nakano, M., Science **157**, 819 (1967).

6. Silvers, W. K., Billingham, R. E., and Sandford, B. H., Nature **220**, 401 (1968).

7. Howard, R. J., Dougherty, S. F., and Mergenhagen, S. E., J. Immunol. **101**, 301 (1968).

8. Taliaferro, W. H. and Taliaferro, L. G., J. Infect Diseases **87**, 37 (1950).

9. Campbell, R. C., *in* "Statistics for Biologists." p. 186. Cambridge Univ. Press, London/New York (1967).

10. Snedecar, G. W. and Cochran, W. G., *in* "Statistical Methods," 6th ed., pp. 130–131. Iowa State Univ. Press, Ames (1967).

11. Dixon, W. J. and Massey, F. J., *in* "Introduction to Statistical Analysis," 2nd ed., p. 294. McGraw-Hill, New York (1957).

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